2021

Stress-Induced Senescence in Human Dermal Fibroblasts : Effects of Creatine and Nicotinamide Post Stress Treatment

Venkata Sravya Arikatla
Wright State University

STRESS-INDUCED SENESCENCE IN HUMAN DERMAL FIBROBLASTS: EFFECTS OF CREATINE AND NICOTINAMIDE POST STRESS TREATMENT

A Thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

by

VENKATA SRAVYA ARIKATLA

Pharm.D., Andhra University, India, 2019

2021

Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

July 27th, 2021

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **Venkata Sravya Arikatla** ENTITLED **Stress-Induced Senescence in Human Dermal Fibroblasts: Effects of Creatine and Nicotinamide Post Stress Treatment** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **Master of Science**.

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Jeffrey B. Travers, M.D., Ph.D.
Thesis Director

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Jeffrey B. Travers, M.D., Ph.D.
Chair, **Department of Pharmacology and Toxicology**

Committee on Final Examination:

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Jeffrey B. Travers, M.D., Ph.D.

_____

Michael G. Kemp, Ph.D.

_____

Ravi P Sahu, Ph.D.

_____

Barry Milligan, Ph.D.
Vice Provost for Academic Affairs
Dean of Graduate School

ABSTRACT

Arikatla, Venkata Sravya, M.S., Department of Pharmacology and Toxicology, Wright State University, 2021. Stress-Induced Senescence in Human Dermal Fibroblasts: Effects of Creatine and Nicotinamide Post Stress Treatment.

In skin connective tissue, dermal fibroblasts appear to be the most prevalent cell type . They are in charge of making the extracellular matrix that makes up the skin's connective tissue, and also involved in wound healing. Moreover, they produce Insulin-like growth factor-1 (IGF-1) which helps in activation of Insulin growth factor-1 receptor (IGF-1R). This receptor helps to control cell proliferation and responses to DNA-damaging substances such as UVB radiation, reactive oxygen species (ROS), and therapeutic drugs. According to our findings, lack of IGF-1 expression in the dermis of elderly patients due to fibroblast senescence (senescence is characterized by which cells enter a condition of irreversible growth arrest after irreversibly avoiding dividing without enduring cell death) has been linked to an increased incidence of skin cancer in the epidermal keratinocyte. Our group resolved that pretreatment with creatine monohydrate and nicotinamide shows a protective effect on oxidative-stress senescence. Based on this study, the present project was designed to study the effect of creatine and nicotinamide on stress-induced reactive oxygen species (ROS) generation as a possible mechanism for their protective effects. Similarly, the present study also examined how the pro-energetics acts on senescence as a post-treatment. Using primary human dermal fibroblasts exposed to H_2O_2 in vitro, via ROS staining, beta-galactosidase staining, and RT-qPCR, we discovered that pre-treatment with creatine and nicotinamide reduces oxidative stress-induced ROS levels, while post-treatment with creatine or nicotinamide after H_2O_2 had no effect on stress-induced senescence.

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ABBREVIATIONS

HDF's - Human dermal fibroblasts

UVR - Ultraviolet radiation

IGF-1 -Insulin-like Growth Factor 1

IGF-1R- Insulin-like Growth Factor Receptor

ROS - Reactive oxygen species

p53 - Tumor suppression protein

p21 - Cyclin-dependent kinase inhibitor 1

SASP - Senescence-associated secretory phenotype

DDR - DNA damage response

ATM - Ataxia-telangiectasia mutated and Rad 3-related

ATP - Adenosine triphosphate

ETC - Electron transport chain

SOD - Superoxide dismutase

Cip-1 - Cyclin-dependent kinase inhibitory protine-1

pRb - Retinoblastoma protein

CDK - Cyclin-dependent kinase

Chk2 - Checkpoint kinase 2

SMS - Senescence-Messaging Secretome

ECM - extracellular matrix

MAPK - Mitogen-activated protein kinase

PI3k - Phosphoinositide 3-kinase

NAM - Nicotinamide

NAD+ - Nicotinamide adenine dinucleotide

NAMPT - Nicotinamide Phosphoribosyl transferase

NMNAT - Nicotinamide-nucleotide adenylyl transferase

NMN - Nicotinamide mononucleotide

NRK - Nicotinamide riboside kinase

Cr - Creatine monohydrate

PCr - Phosphocreatine

AGAT - Arginine glycine amidino transferase

GAA - Guanidino acetic acid

EDTA - Ethylenediaminetetraacetic acid

DCFH2-DA - 2',7'-dichlorodihydrofluorescein diacetate

DMSO - Dimethyl Sulfoxide

HBSS - Hank's Balanced Salt Solution

PBS - Phosphate-Buffered Saline

DNA - Deoxyribonucleic acid

ACKNOWLEDGEMENT

First of all, I would like to thank God for showering his blessings on me throughout my life and giving me the strength for completing the research work.

I am extremely grateful to my mentor Dr. Jeffrey B. Travers, for providing me a chance to work at his laboratory. Thank you so much for helping me with my thesis, providing good comments and a lot of encouragement. I am grateful for his constructive advice, which has made a significant difference in my research. It was a delight to learn about research under his supervision. He had been an excellent guide, assisting with knowledge gaps as needed and emphasizing the significance of teamwork.

I want to thank Dr. Mike Kemp and Dr. Ravi P Sahu for the valuable comments and suggestions that helped me to understand and work productively on my shortcomings in the project. I also want to offer Ms. Christine Rapp my great appreciation for her continuous advice and valuable advice, which have greatly affected my research in my thesis.

I am grateful for the excellent course work that was also helpful during my research and for the great teaching given for the master's program by all the professors. I also thank the Department of Pharmacology and Toxicology.

I am indebted and owe a deep sense of gratitude to my beloved father, mother and family members for their dedicated efforts to educate me to this level and whose unwavering affection and persistent encouragement in saving my career will go throughout their lives. I thank my wonderful friends for their help during my Project.

I would like to dedicate my thesis to my beloved parents, who always encouraged and guided me in all aspects.

Introduction

Statement of problem

Senescence is a cellular response that includes a sustained growth stop as well as other phenotypic changes, such as a proinflammatory secretome. It is involved in normal development, tissue homeostasis, and tumor progression control. On the other hand, it has been linked to a number of age-related diseases (1,2). It can be triggered by a wide range of stressors. These stressors include, both external and internal harmful events, inappropriate cellular growth, oxidative stress, and autophagy factors. The ability of dermal fibroblasts to create the hormone insulin-like growth factor-1 (IGF-1) is decreased in elderly people, according to our group's previous research. As a result, IGF-1R is not activated. We have shown that pretreatment with creatine and nicotinamide protects the dermal fibroblasts from senescence. Then we would like to see if the same impact occurs in post-treatment, which could lead to new treatments for senescence and diseases associated with it. Finally, a goal of these studies was to test the hypothesis that these pro-energetics act by decreasing cellular ROS in response to a pro-oxidative stressor.

Significance

Creatine and nicotinamide reduce ROS levels in stress-induced senescence, protecting dermal fibroblasts from senescence. Previously, we found that pretreatment with creatine and nicotinamide decreases senescence in human dermal fibroblasts (HDF's). Based on these studies, we found that post-treatment might show the same response on senescence. It has the potential to enlighten on how to combat age-related skin disorders.

Statement of purpose

The purpose of this research is to determine if the creatine monohydrate and nicotinamide can protect the stress-induced ROS generation in HDF's. We are also interested to evaluate that post-treatment with creatine monohydrate and nicotinamide can protect human dermal fibroblasts against induction of senescence.

Hypothesis

Creatine and Nicotinamide can protect dermal fibroblasts undergoing stress-induced senescence via protecting against reactive-oxygen species. Hence, post-treatment with those drugs does not show any effect on stress-induced senescence.

Specific Aims

1. Determine the effect of creatine/nicotinamide on H_2O_2-induced ROS generation.
2. Determine the effect of post-treatment with creatine/nicotinamide on IGF-1 production and senescent markers in oxidative stress-induced senescence in dermal fibroblasts.

Literature Review

Aging

Aging is described as the gradual accumulation of major cellular and tissue changes over time, including physiological, structural, and functional alterations, which leads to functional problems and an increased risk of death (3). This degradation is a leading cause of cancer, diabetes, cardiovascular disease, and neurological diseases in humans. Recent advances in aging research have been unparalleled, especially with the finding that the rate of aging is controlled by biological pathways and metabolic mechanisms that have remained constant throughout development.

In general, nine markers of aging may be found, which as shown in Figure-1 are divided into three divisions:

I. Primary Hallmarks.
II. Antagonistic Hallmarks.
III. Integrative Hallmarks.

Primary Hallmarks:

Primary hallmarks include genomic instability, loss of proteostasis, telomere attrition, and epigenetic alterations. These hallmarks are thought to be the primary reason for age-related cell destruction.

Antagonistic Hallmarks:

Antagonistic hallmarks are deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence. These responses first minimize the damage, but if they are persistent or amplified, they eventually become harmful.

Integrative Hallmarks:

Integrative hallmarks consist of stem cell exhaustion and altered intercellular communication. These hallmarks have an impact on tissue function and homeostasis (4,5).

Among all the other characteristics of aging, cellular senescence mechanisms are thought to be the most important component in the intricacy of the process (6).

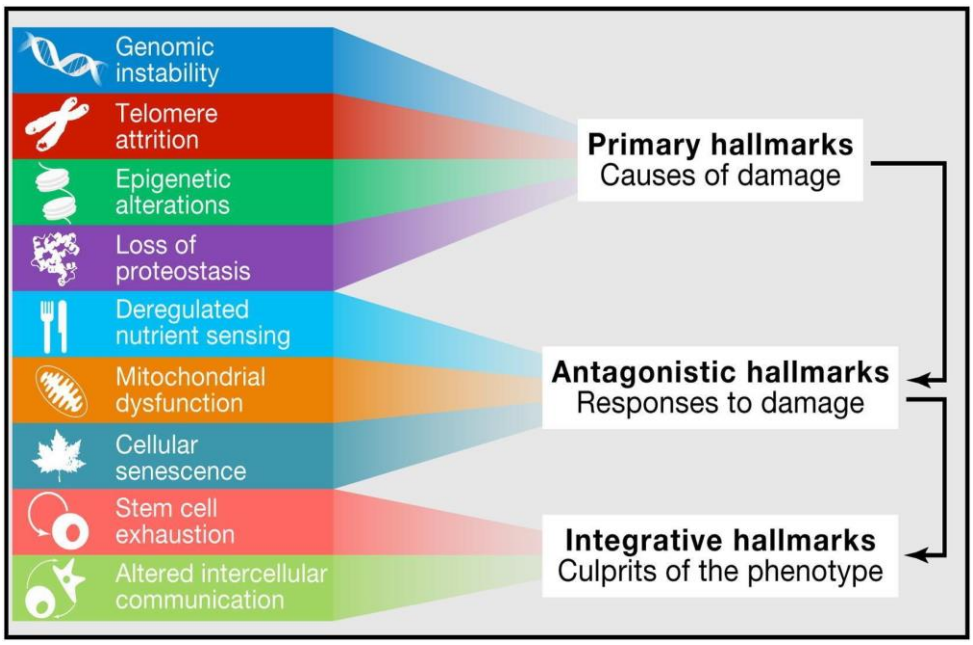


Figure-1 : Interrelationships in Function between the biomarkers of Aging (4)

Cellular Senescence

The term "cellular senescence" refers to a persistent cell cycle arrest in which replicating cells become sensitive to growth-promoting stimuli, usually as a result of DNA damage (7). Leonard Hayflick and Moorhead (1961) were the first to notice that human diploid fibroblasts in culture could only divide a certain number of times before stopping growing. This biological clock, termed as the "Hayflick limit," is triggered by the continual shortening of telomeres with each cell division and is a physiological reaction to prevent genomic instability and, as a result, DNA damage buildup (8). It is now assumed to be a biological response to stress arising from a variety of factors, such as telomere erosion and other DNA damage, mitochondrial failure, neoplastic transformation, and so on. It can be triggered by a variety of conditions that cause the p53/p21 and p16INK4a/Rb pathways to become activated, resulting in an irreversible cell cycle stop. As depicted in Figure-2, cellular senescence occurs in three stages : senescence initiation, early and late senescence. Early phases of senescence are linked to chromatin remodeling, which results in the formation of DNA segments with chromatin changes, promoting senescence and telomere-induced dysfunctional foci, as well as senescence-associated heterochromatin foci. Senescent cells begin to secrete a variety of chemicals, such as chemokines, growth factors, cytokines, and others, which help to determine the senescence-associated secretory phenotype. They improve their mitochondrial

metabolism and become apoptosis resistant. Variations in senescent phenotype and chronic inflammation come from further alterations linked with aging and long-term cellular damage (9). The causes and mechanisms of senescent cells are first described in this study. After that, we talk about the effects of SASP factors and IGF-1 production on aging.

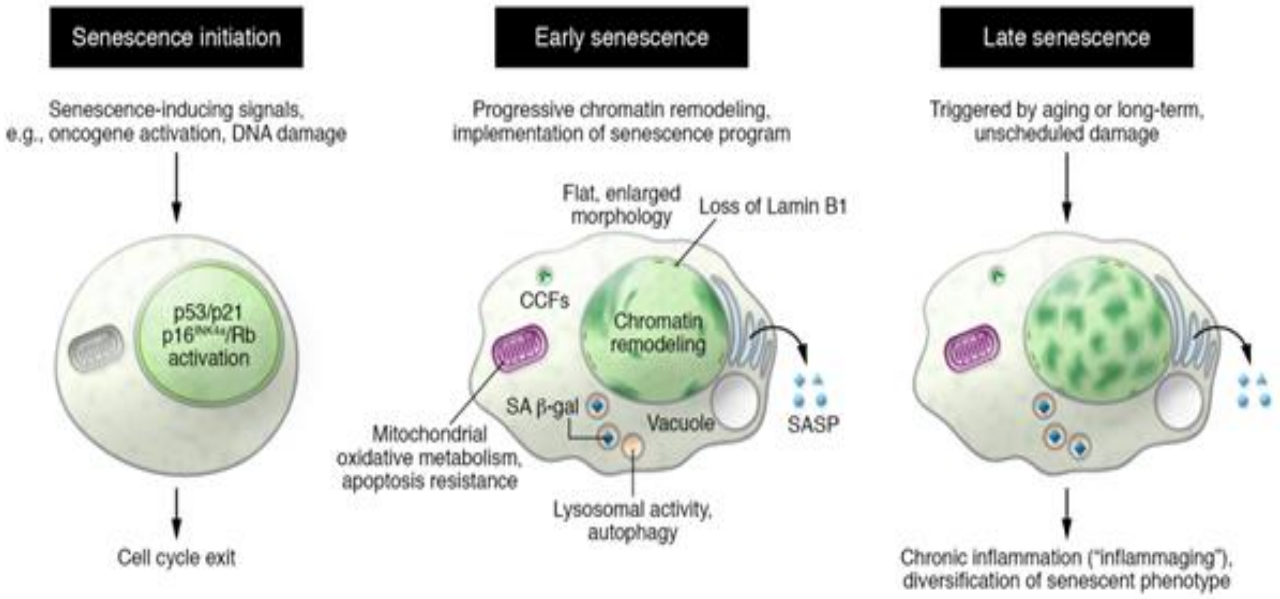


Figure-2 : Schematic illustration of phenotypic changes related with the onset of senescence, early and late senescence phases.(10)

Causes

Senescence is characterized by a sustained cell cycle arrest that is resistant to extrinsic or environmental growth factor activation. As shown in Figure-3, DNA damage response, telomere dysfunction and oxidative stress are the internal and external stressors.

DNA damage response:

Depending on the magnitude of the damage and the physiological setting, DNA damage induces DNA repair, apoptosis, or senescence. Senescent cells have a continuous DNA damage response (DDR), which includes chronic ATM and ATR kinase signaling that leads to cell cycle arrest and senescence via the p53/p21 and p16/pRb pathways. Chemotherapeutics, ionizing radiation, genotoxic stress, and oxidative stress can all cause persistent DNA damage and ultimately senescence (11,12).

Telomere dysfunction:

Telomeres are nucleotide sequences that protect chromosome ends against degradation or fusion with neighboring chromosomes. To compensate for telomere erosion, the enzyme telomerase adds bases to the end of them. Telomerase activity, on the other hand, is insufficient to counteract the high rate of cell proliferation that leads to telomere shortening and aging. Furthermore, telomere erosion activates the DNA damage response, a signaling pathway in which ATM or ATM- and Rad3-related (ATR) kinases stabilize the p53 protein and activate the cyclin-dependent kinase inhibitor p21 through transcriptional activation. In non-proliferating cells, activation of the DDR at the telomeres for a long time, which is a stimulator of cellular senescence, can occur both upon telomere shortening in proliferating cells and following telomeric DNA damage, regardless of telomere length (8,11).

Oncogene activation:

The activation of oncogenes is a potent inducer of cellular senescence. Oncogene expression generates an initial hyperproliferative phase that is organically linked to altered DNA replication, leading to the activation of DDR pathways and senescence. Oncogene-induced senescence is the term for this phenomenon (OIS) (8,11).

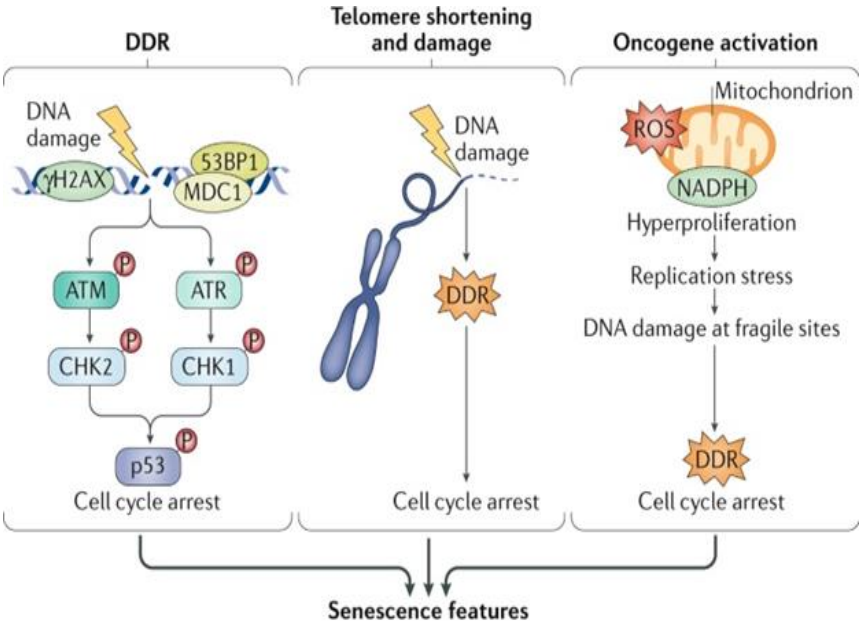


Figure-3 : Diagram shows the phenotypes and drivers of senescence (11)

Oxidative stress:

Reactive oxygen species (ROS) produced by external sources like smoke and radiation, as well as endogenous sources like mitochondria, cause cellular senescence via a DNA damage-response mechanism.

ROS are extremely reactive molecules with a short half-life that are produced when oxygen is partially reduced. Although ROS can come from a variety of places, the majority of them come from electron leakage in the electron transport chain (ETC) in mitochondria. Superoxide anions ($O2^-$), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) are produced when free moving electrons combine with molecular oxygen. Enzymes such as SOD, catalase, and thyroiodin, as well as several small molecule antioxidants, are among the body's defense mechanisms against excessive ROS. Over time, the body's ability to resist excess ROS deteriorates, causing the ageing process to begin. Apoptosis, necrosis, autophagy, and senescence are four cellular fates or phenotypes that can be triggered by excessive ROS in a cell (13).

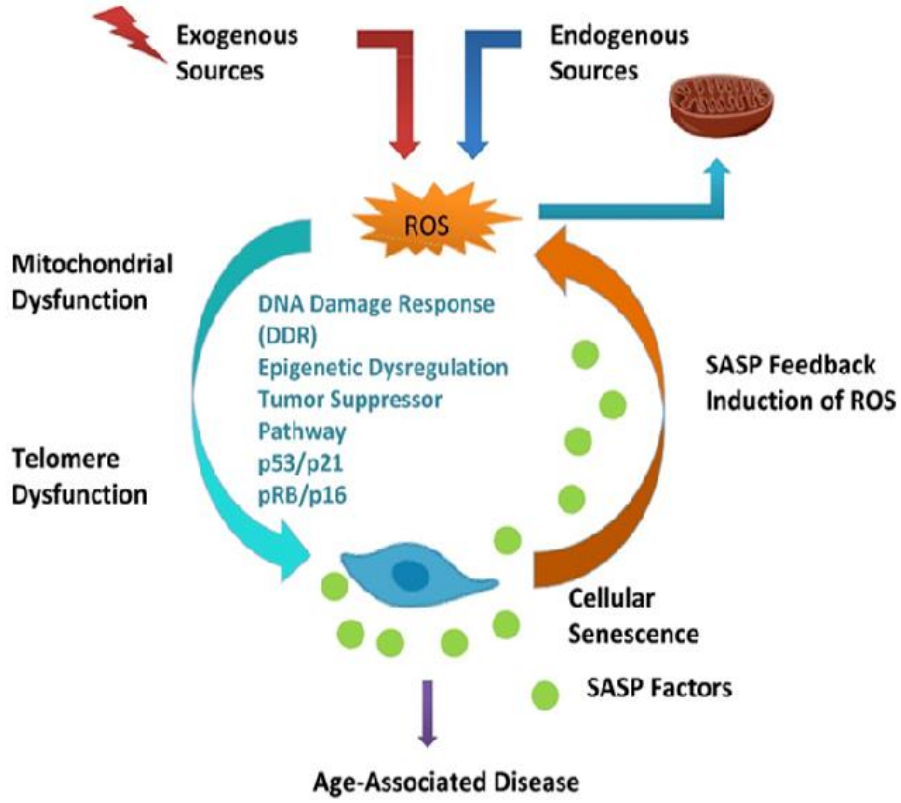


Figure-4 : Sources of ROS generation and leads to age-associated diseases(16)

Pathways

Senescence has been involved in two different pathways (see Figure-5). The activation of either the p53/p21Cip1 or $p16^{INK4A}$/phospho-Rb tumor suppressor pathways cause cell cycle arrest (14). Moreover, p21 and p16 are two CDK inhibitors found in senescent cells and are part of the p53 and Rb controlled tumor suppressor pathways. The expression levels of p21 and p16 are utilized to detect senescent cells in tissues and cultured cells because they are sufficient to generate and maintain the senescence-associated growth arrest. However, not all senescent cell types produce p16, as some tumor cells, particularly those that have lost their Rb activities, might also express it (11).

p53/p21Cip1

In response to DNA damage induced by telomere attrition, oxidative or carcinogenic stress, p53/p21WAF1/Cip1 is activated. Consistent DDR signaling causes p53 to become chronically activated, resulting in cellular senescence (15). As the first member of the mammalian CDK inhibitor family, p21Cip1 is essential for p53-induced cell cycle arrest at either the G1/S or G2/M checkpoints. Once active, p21Cip1 has a variety of roles, including facilitating gene expression control of numerous p53 targets, including CDC25C, CDC25B, and survival, primarily through the recruitment of the E2F4 complex. In addition to p53 action, p21Cip has the ability to induce senescence. Chk2 can promote p-21Cip1 expression in p53-defective cell lines, according to previous studies leading to Chk2-mediated senescence (14,15).

$p16^{INK4a}$/Rb

$p16^{INK4a}$ is a 16kDa protein that binds directly to CDK4/6 and prevents the formation of cyclin D-CDK4/6 complexes, inhibiting Rb phosphorylation and increasing E2F target gene activation. The fact that deletion of the $p16^{INK4A}$ gene or inherited mutations within it has been linked to a variety of human malignancies, including malignant melanoma, demonstrates its importance. This shows that inactivation or deletion of $p16^{INK4A}$ promotes cancer by bypassing senescence (15). The Rb family is one of the most common targets of cyclin-CDK complexes, and its most well-known function is to bind to and inactivate E2F complexes, resulting in transcriptional repression of E2F target genes. When pRb is dephosphorylated, it forms a repressive Rb-E2F complex via binding

to E2Fs. These restrictive complexes attach to the promoter regions of E2F target genes, inhibiting the transcription of cell cycle-related genes (15).

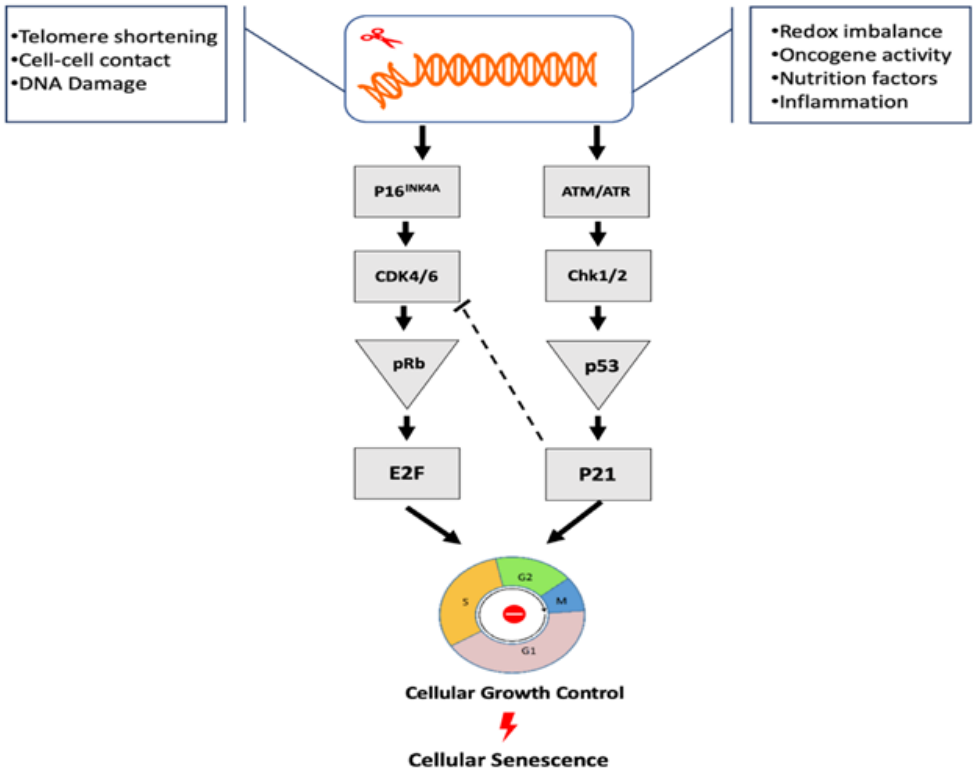


Figure-5 : The Functions of p53 and p16 in Cellular Senescence Regulation (14).

SASP

The senescent cell has the ability to affect the surrounding environment and communicate with adjacent cells by secreting a complex mixture of secreted substances that can change the behavior of surrounding non-senescent cells. One of the fundamental markers of senescence is a hyper secretory phenotype known as the Senescence Associated Secretory Phenotype (SASP) or Senescence-Messaging Secretome (SMS) in which cells suffer dramatic alterations in their secretome (18). The secreted compounds are known to be SASP factors. These SASP factors are categorized into:

A. Soluble signaling factors: (interleukins, chemokines, and growth factors)
B. Secreted proteases
C. Secreted insoluble proteins/extracellular matrix (ECM) components (17).

The auto/paracrine route is how SASP components influence nearby cells. Neighboring cells are also put into a state of cell cycle arrest as a result of this. It's followed by a halt in growth and the onset of senescence (19).

IGF-1

Insulin-like growth factor 1 (IGF-1) is a protein that regulates cell development, division, and proliferation, and its abnormal expression can result in growth abnormalities (21). Dermal fibroblasts release IGF-1, which when coupled to the IGF-1 receptor (IGF-1R) expressed in keratinocytes, stimulates their proliferation as well as a host of other effects which will be reviewed below (20). IGF-1R activation by IGF-1 involves a number of intercellular pathways, including MAPK and PI3K/AKT. Through the activation of the PI3K/AKT pathway, IGF-1 shields keratinocytes from UV-B-induced programmed cell death and supports cell survival via the MAPK pathway (22,23,24,25). Of importance, as reviewed in (22), in the presence of IGF-1 keratinocytes which cannot repair their DNA damage in a timely manner will undergo senescence, which is felt to protect the host from initiated keratinocytes undergoing malignant degeneration. Keratinocytes with IGF-1R in activated state also express higher levels of DNA repair enzymes. The lack of activation of IGF-1Rs in keratinocytes as a result of the decrease in IGF-1 synthesis in the skin could influence the cellular response of keratinocytes to external stress, such as UV radiation, pollution, tobacco smoke, and nutrient deficiencies (26,27,28). A high number of fibroblasts become senescent as people age, resulting in a reduction in IGF-1 synthesis. Furthermore, these keratinocyte responses may have an adverse effect on other intercellular reactions, including cell cycle arrest and death. Skin cancer is caused by a combination of genetic and environmental causes (29).

Nicotinamide

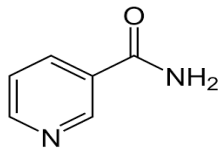


Figure-6 : Niacinamide's chemical structure (49).

Nicotinamide (NAM), a vitamin B3 amide derivative and a key element of the nicotinamide adenine dinucleotide (NAD+) biosynthesis pathway (see Figure-6). Only a little amount of nicotinamide is retained in the liver, with the majority being expelled or catabolized to provide other important metabolic products (31,32). NAD+ is engaged in a variety of biological processes and is a critical regulator of stress tolerance, therefore there is plenty of evidence suggesting that it plays a role in aging. NAD+ levels decrease with aging, causing changes in metabolism and increasing illness vulnerability. It is involved in a number of energy metabolism pathways (33).

NAD+ cannot be imported into mammalian cells in vivo, thus it must be synthesized from tryptophan, or the many forms of niacin consumed in the food, such as nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR). NAD+ is synthesized in cells via three processes, whereas NAM is recycled via the salvage pathway (see Figure-7). The enzyme nicotinamide phosphoribosyl-transferase (NAMPT) catalyzes the conversion of nicotinamide to NMN, which is the first step in the salvage pathway. The nicotinamide mononucleotide adenylyl transferase (NMNAT) enzyme then converts NMN and ATP into NAD+. The nicotinamide riboside kinase (NRK) enzyme can convert NR to NMN, which is involved in the salvage pathway. In the salvage pathway, NAMPT is the rate-limiting enzyme. Reduced NAD+ synthesis, which may be owing to decreasing NAMPT activity, has been proposed as one of the causes of lower NAD+ levels with age. NAMPT levels have been shown to decrease with age in a variety of tissues, but exercise enhances NAMPT expression in skeletal muscle (30,31,33,34).

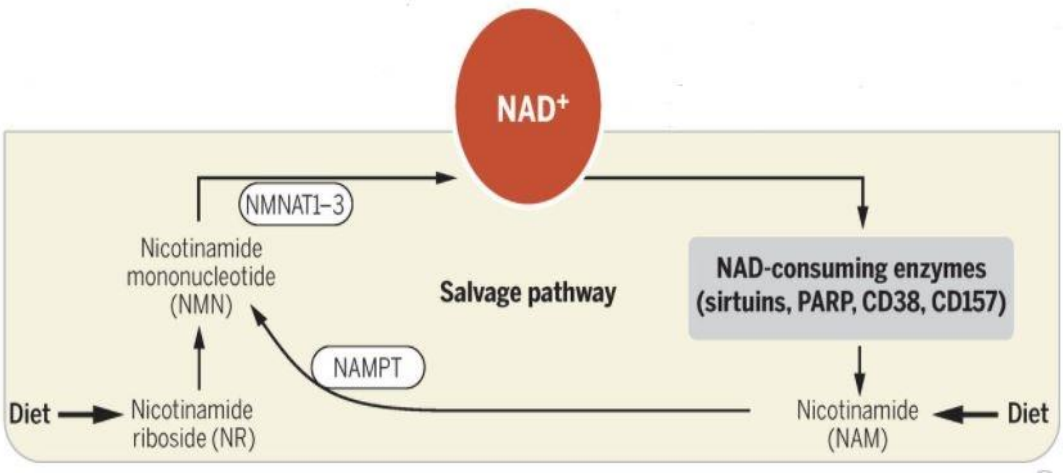


Figure-7 : Salvage pathway for NAD+ biosynthesis (30).

Creatine

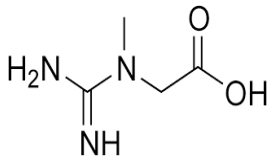


Figure-8 : Creatine chemical structure.

Creatine is a naturally occurring organic acid (see Figure-8) that is endogenously generated in the kidneys and liver via processes involving the amino acids arginine, glycine, and methionine (40). Depicted in Figure-9, on a daily basis, roughly 1-2 grams are produced, with about 95% of it being exported and stored in skeletal muscle as phosphocreatine (PCr). The brain, testes, and kidney store the remaining as unbound free creatine (35). Intramuscular creatine reserves are broken down and eliminated in the urine as creatinine at a rate of about 2 g/day (1–2%). To replace lost creatine, both exogenous dietary intake and endogenous de novo synthesis is employed. Dietary intake such as meat and fish. Therefore, meats containing roughly 3-4 grams of creatine per kilogram of meat are consumed (37). In the liver and kidney, endogenous de novo creatine production takes place in two stages. The enzyme arginine glycine amidinotransferase (AGAT) first converts arginine and glycine to ornithine and guanidino acetic acid (GAA). Second, the methyl group from S-adenosyl methionine is transferred to guanidinoacetate to produce creatine (38,39,40). Cr appears to have a protective effect on physical strength, muscle mass, and cognitive functioning as people age, according to the literature (37). It has been linked to improvements in a variety of ailments, including myopathies, neurological disorders, rheumatic diseases, and type 2 diabetes (41,42,43,44). Furthermore, Cr supplementation has an outstanding safety profile following hundreds of published trials and millions of exposures (36).

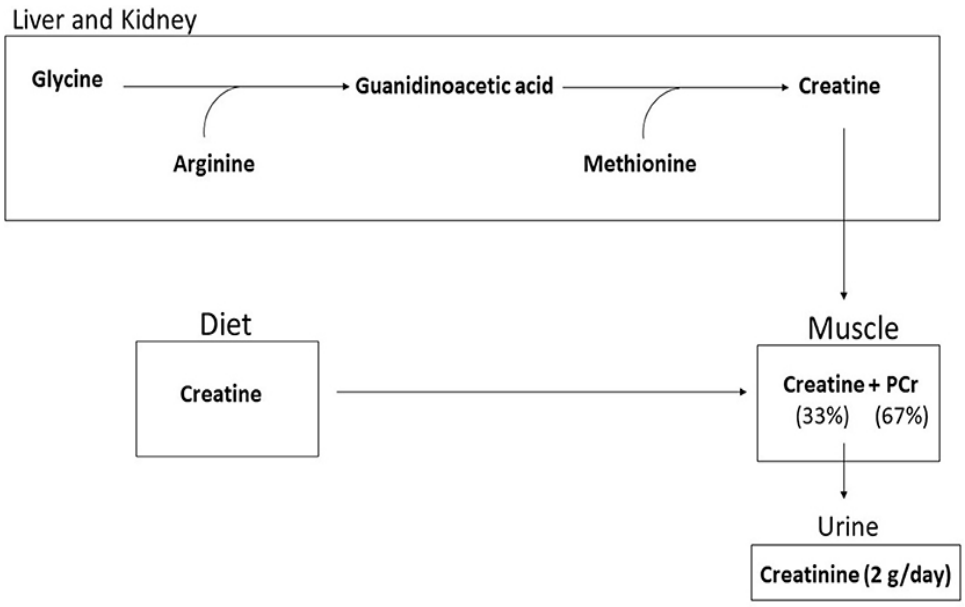


Figure-9 : Synthesis of creatine endogenously (35).

MATERIALS AND METHODS

Introduction

This chapter addresses the experimental models, materials, procedures, and analysis used to produce relevant data in accordance with the study's goals and objectives. In this research, in vitro cell lines were used as a basic model.

Cell culture

Primary cell lines were used in this study. Neonatal human dermal fibroblasts (NHF) were derived from the human skin (discarded foreskins). These are flat, large, spindle-shaped (elongated) cells and mostly involved in connective tissue. We obtained these cells from Dr. Dan Spandau from Indiana University.

Cell growth media and storage conditions

Neonatal human dermal fibroblasts (NHF) were cultured in Dulbecco's modified Eagle's medium (DMEM) media. A 500 ml bottle of DMEM low glucose with 50 ml of 10% FBS FetalClone III, 5 ml of 2 mM glutamine and 5 ml of 100 U penicillin/ 0.1 mg/ml streptomycin were used as a media. All the cells were incubated at 37^{o}C in an incubator which maintained 20% O_2 and 5% CO_2 levels.

Defrost the cells

Cells were defrosted by placing the frozen cells from the cryovial in a 37^{o}C water bath as soon as possible after removing it from the liquid nitrogen storage. Swirling the vial gently until only a small amount of ice remains then the cells are seeded in the 100 mm plate containing 10 ml of complete growth media. The media was changed after 24 hrs.

Changing media

Old media was discarded from the culture plate and washed twice with 1X PBS prior to changing the media. Then 10 ml of fresh complete media was added to the plate. Between passing, the media was changed every two days.

Cell passage

After ~80% of confluency, the media was discarded from the plate and the cells washed with 1X PBS for 2-3 times. Then 2 ml of 0.05% Trypsin-EDTA 1X was added and the plate was placed in the incubator for 3-5 minutes. After the cells were separated from the plate, 8 ml of DMEM low glucose media was added to each plate to make the volume 10 ml, and the cells were triturated. Then 2 ml of cell sample was transferred into a 100 mm new plate and made the volume to 10 ml by adding 8 ml of media. Prior to treatment, the cells were counted and added to new plates to ensure that each plate had an equal number of cells. Cells were counted, grown and trypsinized at regular intervals to ensure consistency.

Cell count

To get the cell count/ml we have to wash the cells with 1X PBS for 2-3 times. Then add 2 ml of 0.05% Trypsin-EDTA 1X and keep the plate in the incubator for 3-5 minutes. 8 ml of media was added as soon as the cells were separated. In 10 µl of Trypan blue stain, 10 µl of this suspended cell mixture was added. The cell count was obtained on the countess machine using 10 µl of the resulting mixture pipetted onto a slide. Before plating, the average of two reading counts was taken to obtain accurate values. For my experiments, I have plated 200,000 cells in each 100 mm and 60mm cell culture plates, 100,000 cells in each 60 mm cell culture plates and 30,000 cells in each 35 mm cell culture plates.

Treatment

Based on the treatment groups, cells were divided into different plates. Apart from DMEM low glucose media, the control plate did not receive any treatment. Sterilized ultrapure water was used to treat the vehicle treatment group.

DCFH2-DA

Stock solution of 100 mM DCFH2-DA (487.29 g/mol) was prepared in DMSO. Stock solution was added directly to the serum free DMEM low glucose media to get the desired concentration level.

Hydrogen peroxide

Using the previous data, I used a concentration of 600 µM in my experiments. To reach the desired concentration of H_2O_2 (600 µM) was added directly to the low glucose media.

Nicotinamide

In 10 ml of warm sterilized ultrapure water, a stock solution of 100 mM creatine monohydrate (149.15 g/mol) was prepared. The powder form will dissolve better in warm water than in cold water. Finally, 0.22 µm syringe filter was used for the sterilization and good to prepare fresh stock solution for each experiment.

Creatine monohydrate

Alternatively, 10 ml of 100 mM nicotinamide (122.12 g/mol) stock solution was prepared with autoclaved ultrapure water. For sterilization, 0.22 µm syringe filter was used, and fresh stock solution was prepared for each experiment.

Experimental design

Cells were treated with 600 µM H_2O_2 because the use of H_2O_2 to cause oxidative damage/stress in cellular models is a common procedure (45). For my experiments, I have plated 200,000 cells in each 100 mm cell culture plates and 30,000 cells in each 35 mm cell culture plates. After 48 hrs of incubation, the cells were washed with PBS (1X) and treated with H_2O_2 for 2 hrs. Later, cells were washed with PBS (1X) for 2-3 times and added fresh media along with vehicle, creatine (5 mM, 10 mM), nicotinamide (2.5 mM, 5 mM) and combination of 5 mM nicotinamide + 5 mM creatine as a post-treatment. After 72 hrs of incubation, the cells were used in additional experiments. In this study, a total of eight post-treatment and two pretreatment groups were used.

For the measurement of ROS, 200,000 cells were plated in a 60 mm plate.

Treatment groups

- **Control group:** Only DMEM low glucose media was used to treat the cells.
- **Vehicle:** Sterilized water is used as a vehicle.
- **Hydrogen peroxide group:** Cells were treated with 600 µM of H_2O_2.

- **Nicotinamide treated group:** (2.5, 5) mM concentrations of nicotinamide were used to treat the cells.
- **Creatine monohydrate:** (5, 10) mM concentrations of creatine were used to treat the cells.
- **Creatine monohydrate and nicotinamide:** Creatine monohydrate and nicotinamide (5 mM each) were added to the cells.

Evaluation of the intracellular ROS levels

To measure intracellular ROS, the non-fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate was used (DCFH2-DA; Sigma-Aldrich Co., LLC, USA). 200,000 cells were cultured in 60 mm plate, pretreated for 24 hrs with DMEM containing various concentrations of creatine and nicotinamide, washed twice with HBSS and incubated for 30 minutes in the dark at 37°C with 5 µM of DCFH2-DA. The cells were then washed 3-4 times with HBSS before being incubated with H_2O_2 for 30 minutes. Then, the cells were washed twice more with HBSS before being examined under a fluorescence microscope using the BioTek Cytation 5 machine (46) and the mean fluorescence intensity was measured by using image j software.

Senescence-associated β-galactosidase (SA-β-gal) staining

This staining was performed after 72 hrs post-treatment to look for the biomarker of senescence using an Abcam senescence detection kit.

Reagent preparation

To prepare a 20X stock solution, weigh 20 mg X-gal and dissolve in 1 mL DMSO or DMF. Excess X-gal solution can be kept at -20°C (light-protected) for one month. When making and storing the X-gal, always use a polypropylene container or glass. Polystyrene is not to be used.

Sample preparation

After removing the culture medium, wash the cells once with 1X PBS. Then, fix the cells in 1 ml of fixative solution at room temperature for 10-15 minutes. Prepare the staining solution Mix using a polypropylene plastic tube while the cells are in the fixative solution. Make enough solution to stain the number of wells. Prepare 940 µl of staining solution, 10 µl of staining supplement, and 50 µl of DMSO containing 20 mg/ml X-gal. Wash the cells twice with 1X PBS before adding 1 ml of the staining solution mix to each well. Cover the plate with paraffin paper and incubate the

plate overnight at 37°C in a CO_2-free incubator. The following day, examine the cells under a microscope for the development of blue color using the BioTek Cytation 5 machine.

RT-qPCR

For this experiment, 200,000 cells were plated in 100 mm cell culture plates.

Reagent preparation

Buffer RLT plus: Add 10 µl of β-mercaptoethanol (β-ME) per 1 ml Buffer RLT Plus if purifying RNA from RNase-rich cell lines. Buffer RLT Plus with β-ME can be kept at room temperature (15–25ºC) for up to 1 month.

Buffer RPE: It is delivered in the form of a concentration. To obtain a working solution, add 44 ml of 100% ethanol, as directed on the bottle, before using the first time.

Purification of total RNA from cells

After 72 hrs of post-treatment, the media was discarded and washed with 1X PBS. Then add 2 ml of 0.05% Trypsin-EDTA 1X and the plate was placed in the incubator for 3-5 minutes. After the cells were separated from the plate, 8 ml of DMEM low glucose media was added to each plate to make the volume 10 ml, and the cells were triturated. The cells containing media were collected in the 15 ml tube and centrifuged for 5 minutes at 1500 rpm. Carefully remove the media and wash the cells with PBS, centrifuged for 5 minutes at 3000 rpm. Remove the supernatant and collect the cell pellet (Note: Cell pellet can be stored at -80ºC for later use). RNA was isolated from the cell samples using RNeasy Plus Mini Kit (Qiagen Cat No. 74134). Disrupt the cell pellet by adding 350 µl of RLT plus and pipet to mix. Then pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged at maximum speed for 2 minutes at 4ºC. Transfer the homogenized lysate to a 2 ml collection tube containing a gDNA elimination spin column. Discard the flow-through. Then, 700 µl of buffer RW1 was added to the RNeasy spin column and centrifuged for 15 sec at ≥8000 x g to wash the spin column membrane. The flow-through was discarded. 500 µl of buffer RPE was added to the RNeasy spin column and centrifuged for 15 sec at ≥8000 x g. Discard the flow-through. Again, 500 µl of buffer RPE was added to the RNeasy spin column and centrifuged for 2 minutes at ≥8000 x g. Placed the RNeasy spin column in a new 2 ml collection tube and centrifuged for 1 minute at full speed. Collect the RNeasy spin column

and place it in a 1.5 ml collection tube. To elute the RNA, add 30-50 µl of RNase-free water to the spin column and centrifuged for 1 minute at ≥8000 x g. To measure the RNA concentration (ng/µl) and purity (A260/280 ratio should be 2.0) a Nanodrop spectrophotometer was used.

Reverse Transcription

For reverse transcription, QuantiTect Reverse Transcription Kit (Qiagen: 205311) was used. After determining the RNA concentration, add the desired volume of RNA to a PCR tube and make up the volume up to 12 µl by adding RNase-free water. 2 µl of 7X genomic DNA buffer was added and incubated for 2 minutes at 42ºC using a thermal cycler. Place the samples on ice and set aside. For reverse transcription master mix, it contains 4 µl RT buffer, 1 µl Primer and 1 µl RT enzyme for one reaction. Add 6 µl of master mix to each sample and bring the total volume to 20 µl. Then the samples were incubated at 42ºC for 15 minutes and then incubated for additional 3 minutes at 95ºC. The RT reactions can be stored at -20ºC until the use of Q-PCR.

Q-PCR

I usually prepare 10 µl reactions and run Q-PCR in triplicate. 5 µl of 2X TaqMan Fast Universal PCR Master Mix, 0.5 µl of 20X TaqMan primer/probe set (FAM), 0.5 µl of RT reaction, 4.0 µl of molecular biology grade water for a single reaction Make a master mix for the number of reactions required. 9.5 µl of the master mix and 0.5 µl RT reaction should be aliquoted into each of the 96-well plate. The reference gene used in this study is Beta-2-microglobulin and the gene of interest varies. The plate was run; the thermal profile included a 3-minute melting step at 95 °C, followed by 10 seconds at 95 °C and 30 seconds at 55 °C through 40 cycles on the BioRad CFX96 Q-PCR machine. Ct values were obtained from the software and the expression fold change was calculated using the ΔΔCt method in comparison to the no-treatment group.

Statistical analysis

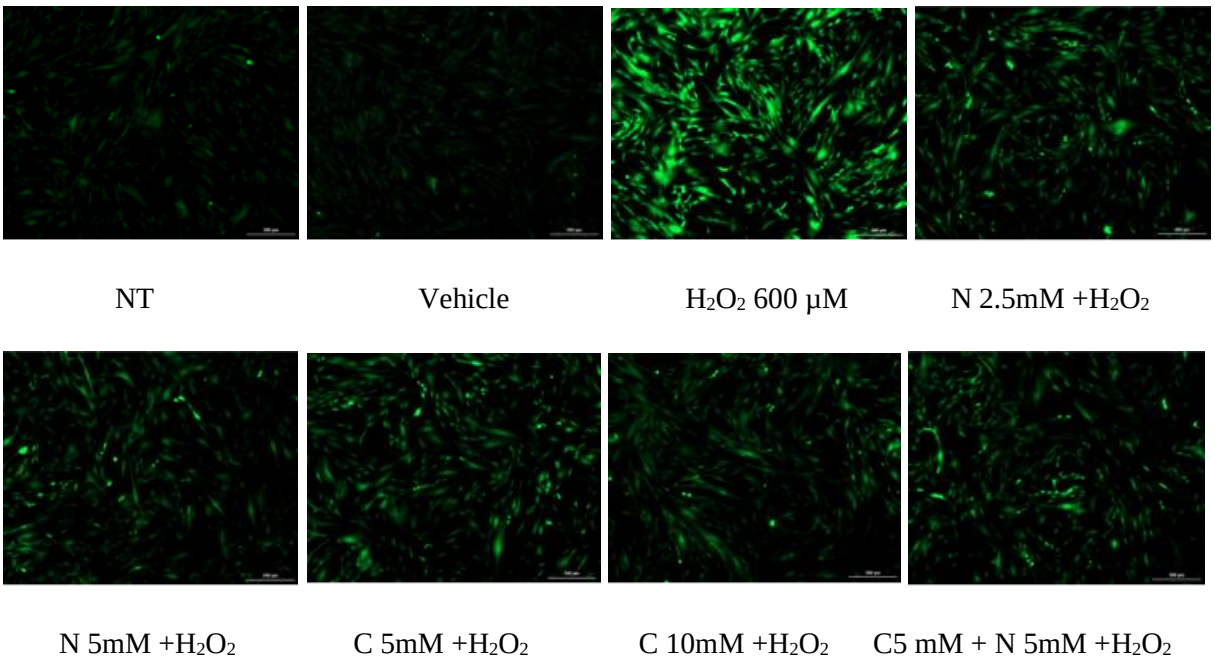
Analysis has been performed by using the GraphPad prism. The One-way ANOVA test was used for comparing the two groups. The data is presented as the average of at least three experiments with standard Errors (S.E.). The differences between the samples were judged significant when the P values were less than 0.05. If $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (***) are the significant levels, respectively.

RESULTS

Creatine/Nicotinamide decreases H_2O_2-induced ROS accumulation in HDF,s

We assessed intracellular ROS levels in H_2O_2 exposed HDF cells in this study using the DCFH2-DA fluorescent technique. Figure-10 shows that when compared to the NT (No Treatment) group, the H_2O_2 treated group dramatically raised intracellular ROS, but NAM 2.5 mM + H_2O_2, NAM 5 mM + H_2O_2, Cr 5 mM + H_2O_2, Cr 10 mM + H_2O_2 and combination therapy (Cr 5 mM + NAM 5 mM + H_2O_2 considerably decreased ROS levels.

(A)



NT | Vehicle | H_2O_2 600 µM | N 2.5mM +H_2O_2

N 5mM +H_2O_2 | C 5mM +H_2O_2 | C 10mM +H_2O_2 | C5 mM + N 5mM +H_2O_2

(B)

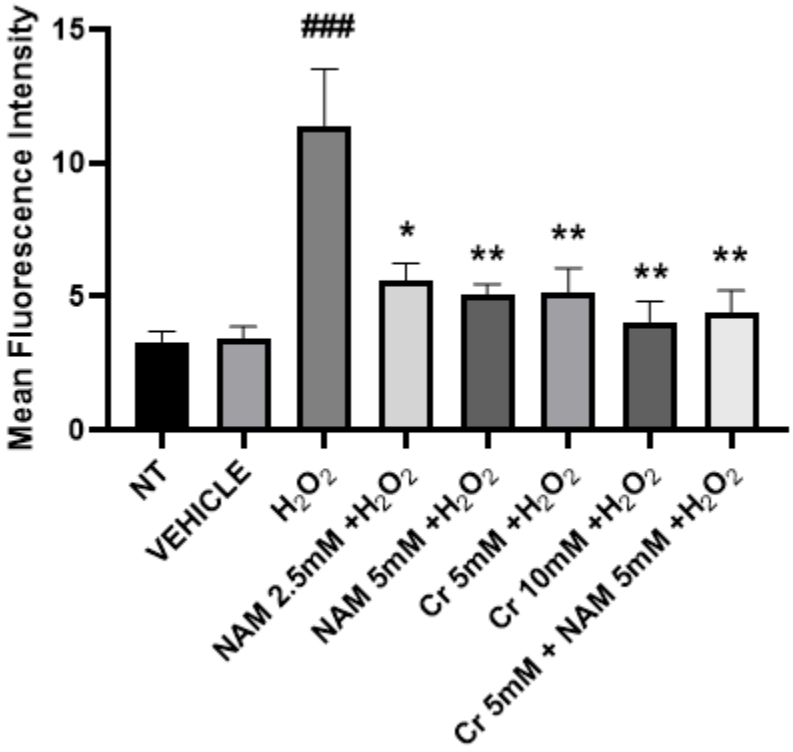


Figure-10: Creatine/Nicotinamide reduces H_2O_2-induced ROS production in HDF's. NHF cells were incubated with or without Cr/NAM (2.5mM, 5mM, 10mM) for 24 hrs. After the cells were washed and incubated with 5 µM DCFH2–DA for 30 mins, followed by 30mins 600 µM H_2O_2 treatment. (A) Fluorescence microscopy of cells (B) Fluorescence intensity was measured by using image J software. The data is shown as the mean ± SE of three experiments. One-way ANOVA and Tukey's post-hoc test was used to compare groups. $P < 0.0001$ (####) compared with NT grouped cells, ($P < 0.05$*, $P < 0.01$**) compared with H_2O_2 treated cells.

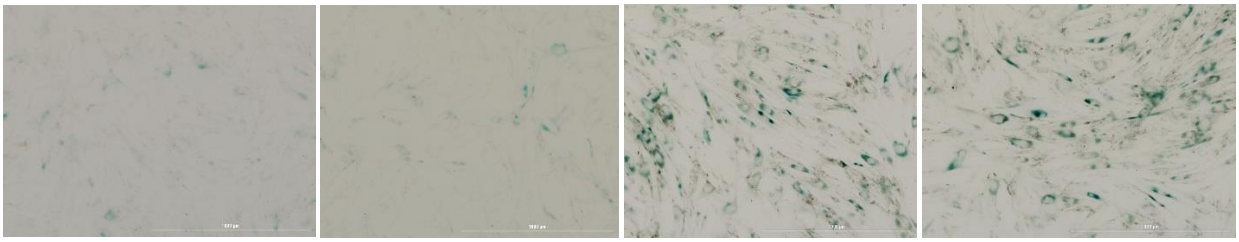
Effect of Creatine on Stress-induced senescence

Post treatment with Creatine on H_2O_2 induce SA-β-gal-positive cells

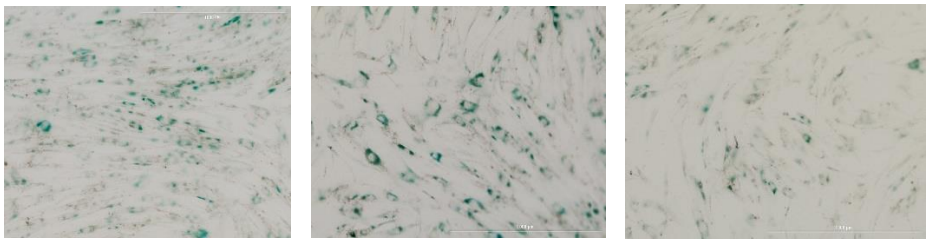
To determine the effect of Cr on beta gal activity, cells were treated with H_2O_2 for 2 hrs. Later, cells were washed with PBS (1X) for 2-3 times and added fresh media along with creatine (5 mM,

10 mM) and a combination of Cr +NAM (5 mM+5 mM) as a post-treatment. SA-β-gal staining was performed after 72 hrs of incubation. The results demonstrated that the H_2O_2 treated group dramatically raised the percentage of beta gal positive cells when compared to the NT group, but the cells which are treated with Cr 5 mM + H_2O_2, Cr 10 mM + H_2O_2 and Cr 5 mM + NAM 5 mM + H_2O_2 does not show any significance difference compared to the H_2O_2 treated group.

(A)



NT Vehicle H_2O_2 H_2O_2+ Cr 5mM



H_2O_2+ Cr 10mM H_2O_2+ Cr 5mM+ N 5mM Cr 10mM +H_2O_2

(B)

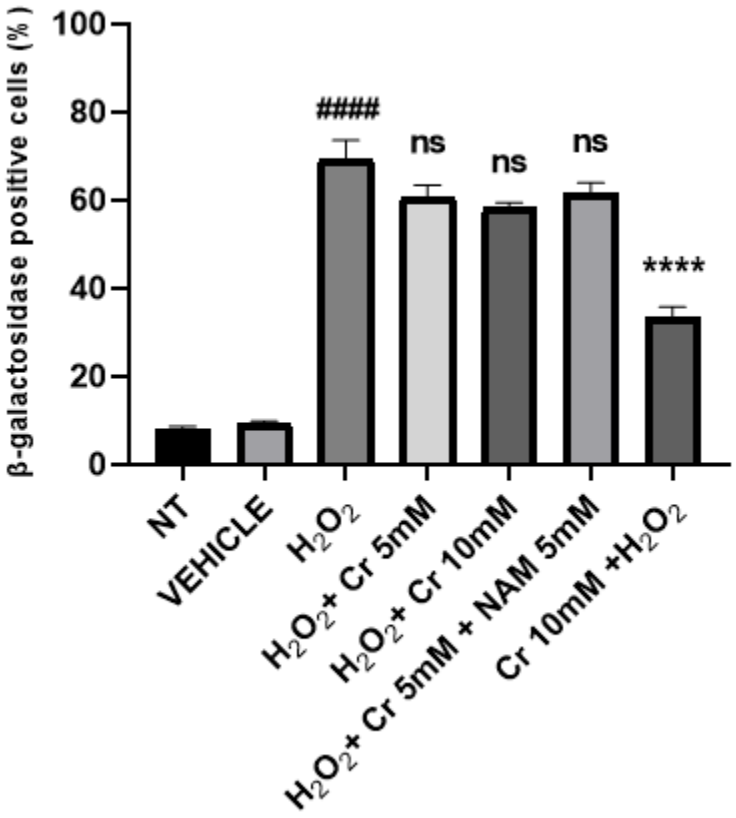


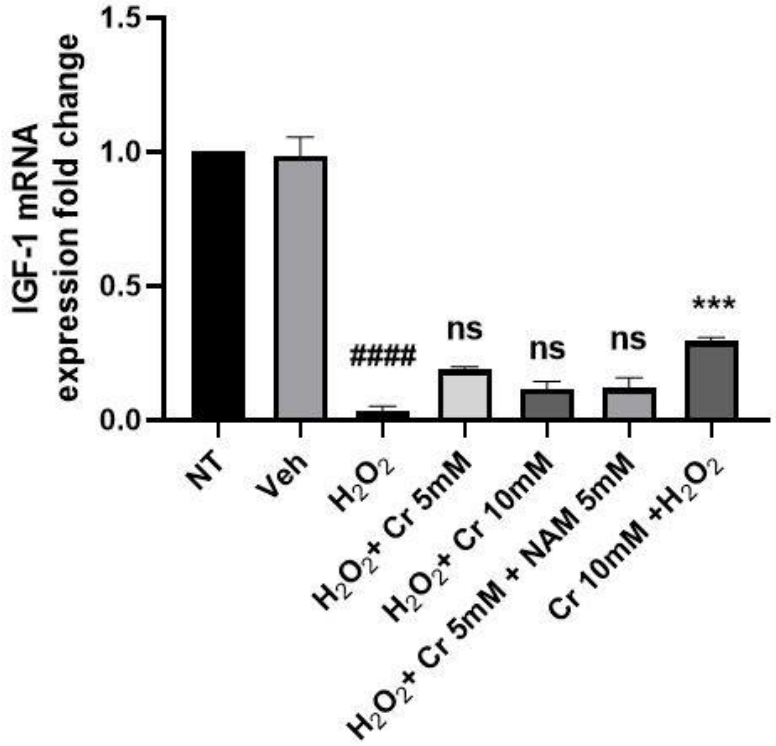
Figure-11: Senescence-associated-β-galactosidase (SA-β-gal) staining in HDF's. The H_2O_2 treated group had a higher number of SA-gal+ cells. The fraction of SA-gal+ cells in the H_2O_2 + Cr (5 mM, 10 mM) and a combination of Cr+ NAM (5 mM+5 mM) groups do not show any significant difference compared to the H_2O_2 group but the pretreatment with Cr shows significant difference compared to H_2O_2 treated cells. The data is shown as the mean ± SE of three experiments. One-way ANOVA and Tukey's post-hoc test was used to compare groups. $P < 0.0001$ (####) compared with NT grouped cells, $P < 0.0001$ (****), ns (non-significance) compared with H_2O_2 treated cells.

Effect of Creatine post treatment on IGF-1 and SASP mRNA expression

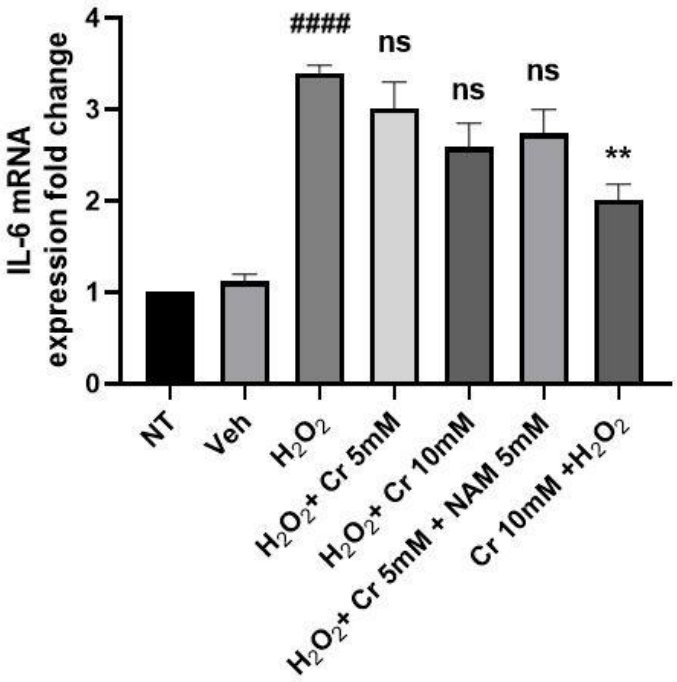
To determine whether the post treatment of Cr shows an effect on mRNA expression, we isolated the RNA from the treated cells and RT-qPCR was performed to measure the mRNA expression

for IGF-1, IL-6, IL-8 and TNF-α. As show in the Figure-12, the H_2O_2-treated group shows significant decrease in IGF-1 mRNA expression fold change when compared to NT group, but there is no significance between H_2O_2+ creatine (5mM, 10mM), combination of H_2O_2+ Cr + NAM (5mM+5mM) and H_2O_2 treated groups. As well we can see H_2O_2 treated group shows significant increase in SASP (IL-6, IL-8 and TNF-α) mRNA expression fold change when compared to NT group, but there is a significance between creatine (5mM, 10mM) +H_2O_2 and H_2O_2 treated groups

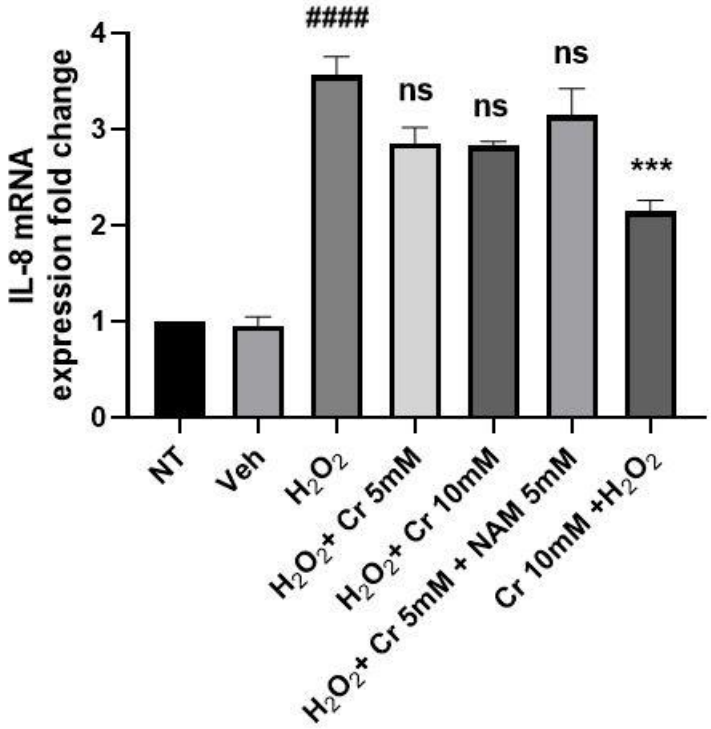
(A)



(B)


IL-6 mRNA expression fold change
0
1
2
3
4
####
ns
ns
ns
**
NT
Veh
H2O2
H2O2+ Cr 5mM
H2O2+ Cr 10mM
H2O2+ Cr 5mM + NAM 5mM
Cr 10mM +H2O2

(C)


IL-8 mRNA expression fold change
0
1
2
3
4
####
ns
ns
ns
***
NT
Veh
H2O2
H2O2+ Cr 5mM
H2O2+ Cr 10mM
H2O2+ Cr 5mM + NAM 5mM
Cr 10mM +H2O2

(D)

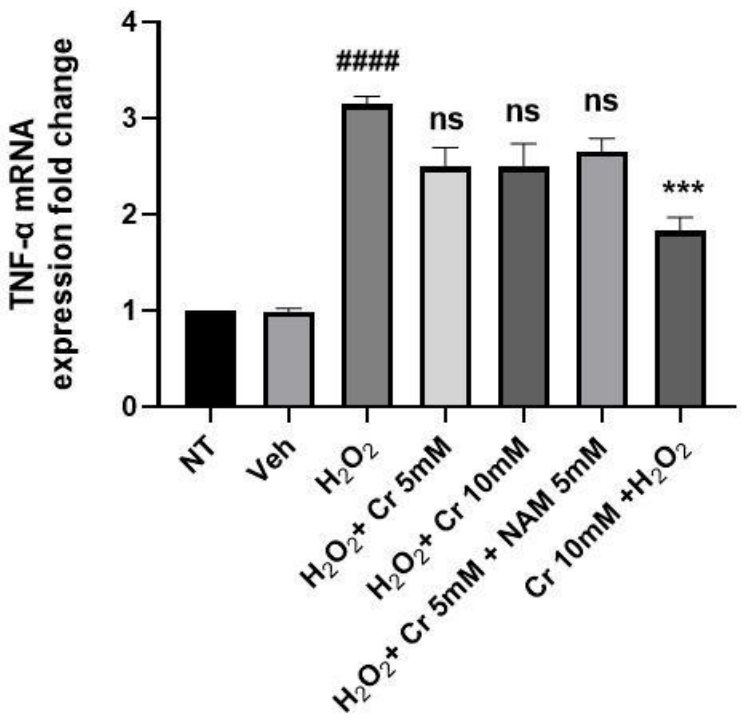


Figure-12: Effect of Creatine post treatment on IGF-1 and SASP mRNA expression. RNA was extracted from NT, H_2O_2, and Cr-treated cells, and RT-qPCR was conducted. The mean ± S.E of three experiments relative mRNA expression of (A) IGF-1, (B) IL-6, (C) IL-8, and (D) TNF-alpha is represented in the data. The One-Way ANOVA and Tukey's post-hoc test was used to compare groups. If the P value was less than 0.05, differences between samples were considered significant. P < 0.05 (####) differences between NT and H_2O_2 treated cells; P < 0.05 (*) P < 0.01 (**), P < 0.001 (***) and P < 0.0001 (****)differences between H_2O_2 and Cr treated group

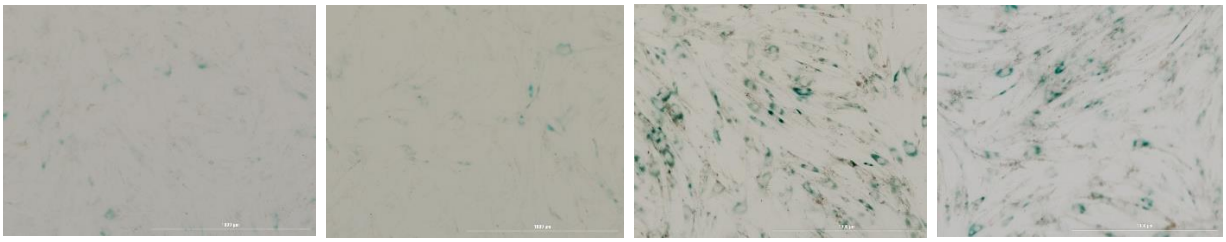
Effect of Nicotinamide on Stress-induced senescence

Post treatment with Nicotinamide on H_2O_2 induce SA-β-gal-positive cells

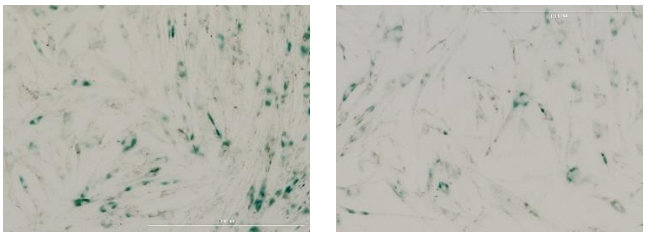
To determine how NAM affects beta gal activity, cells were exposed to H_2O_2 for 2 hrs. After that, the cells were rinsed with PBS (1X) for 2-3 times before being given fresh medium and a post-

treatment of NAM (2.5mM, 5mM). After 72 hrs of incubation, SA-gal staining was conducted. When compared to the NT group, the H_2O_2 treated group significantly increased the percentage of beta gal positive cells, however the cells treated with H_2O_2+ NAM (2.5 mM, 5 mM) showed no significant difference when compared to the H_2O_2 treated group. We can also see that NAM +H_2O_2 (5 mM) shows a significant difference compared to H_2O_2 treated cells.

(A)



NT Vehicle H_2O_2 H_2O_2+ NAM 2.5mM



H_2O_2+ NAM 5mM N 5mM +H_2O_2

(B)

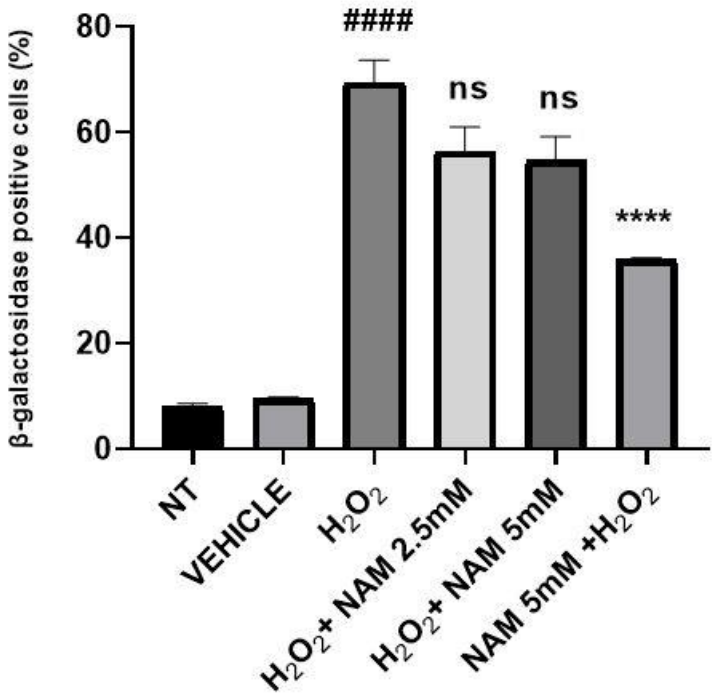


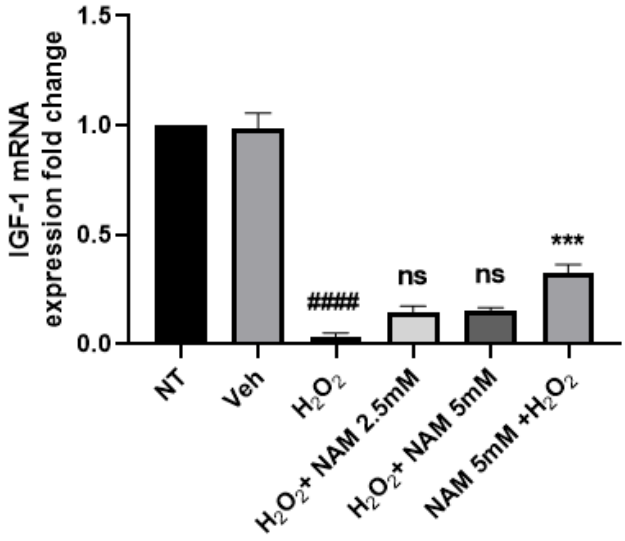
Figure-13: Effect of NAM on Senescence-associated-β-galactosidase (SA-β-gal) staining in HDF's. The H_2O_2 treated group had a higher number of SA-gal+ cells. The fraction of SA-gal+ cells in the H_2O_2 + NAM (2.5mM, 5mM) group do not show any significant difference compared to the H_2O_2 group, but the pretreatment with NAM shows significant difference compared to H_2O_2 treated cells. The data is shown as the mean ± SE of three experiments. One-way ANOVA and Tukey's post-hoc test was used to compare groups. $P < 0.0001$ (####) compared with NT grouped cells, $P < 0.0001$ (****), ns (non-significance) compared with H_2O_2 treated cells.

Effect of Nicotinamide post treatment on IGF-1 and SASP mRNA expression

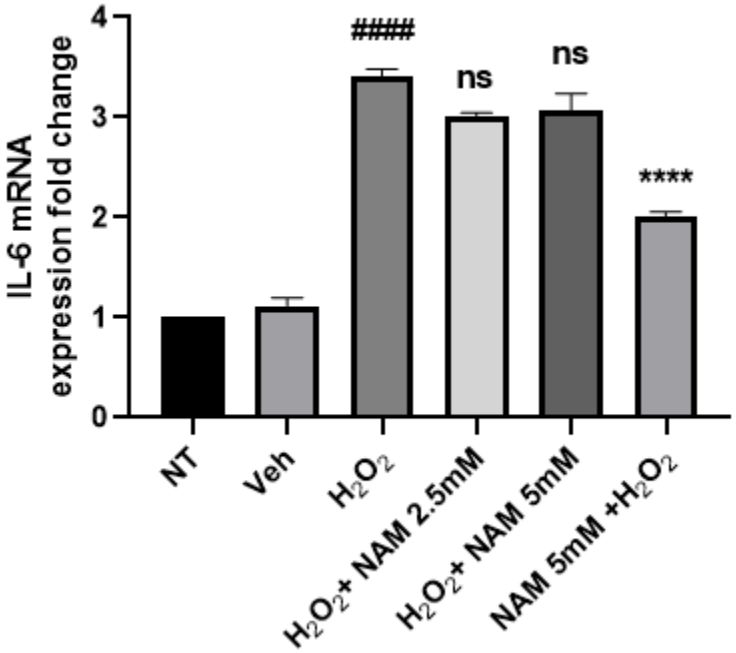
We extracted RNA from the treated cells and used RT-qPCR to evaluate the mRNA expression for IGF-1 and TNF-α to determine if the NAM treatment had an influence on mRNA expressions. The H_2O_2 treated group has a significantly lower IGF-1 mRNA expression fold change than the NT group, as seen in the figure-14, but there is no difference between the H_2O_2+ NAM (2.5 mM,

5 mM) and H_2O_2 treated groups. As well we can see H_2O_2 treated group shows significant increase in SASP (IL-6, IL-8 and TNF-α) mRNA expression fold change when compared to NT group, but there is significance between NAM (2.5mM, 5mM)+ H_2O_2 and H_2O_2 treated groups.

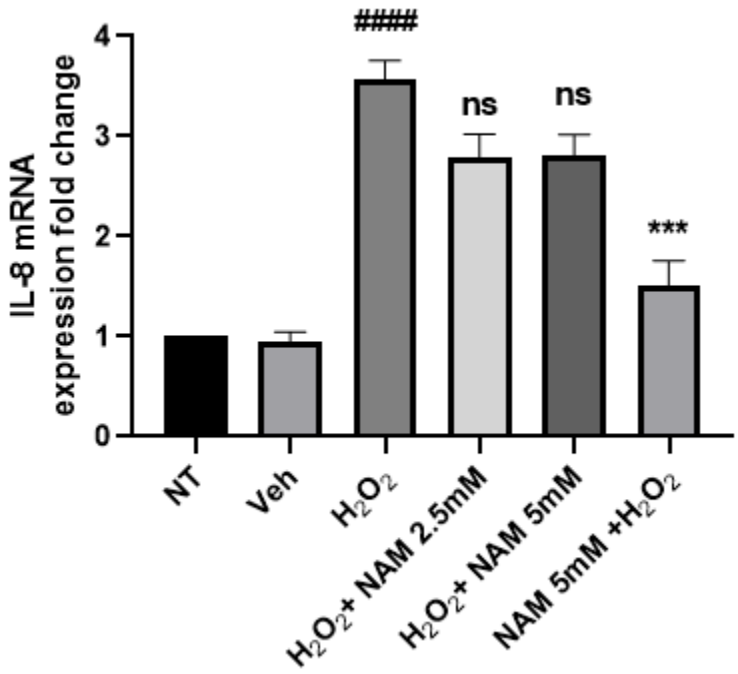
(A)



(B)



(C)


IL-8 mRNA expression fold change
0
1
2
3
4
####
ns
ns
***
NT
Veh
H2O2
H2O2+ NAM 2.5mM
H2O2+ NAM 5mM
NAM 5mM +H2O2

(D)

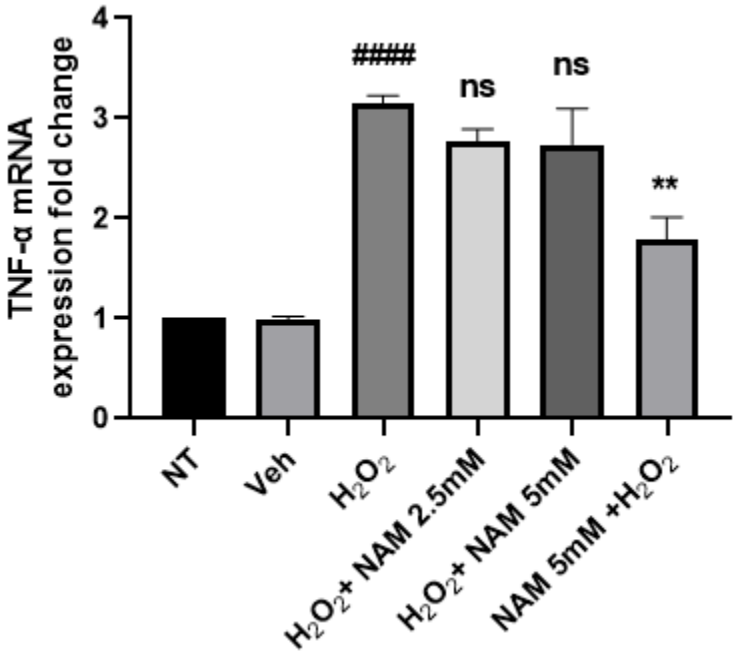

TNF-α mRNA expression fold change
0
1
2
3
4
####
ns
ns
**
NT
Veh
H2O2
H2O2+ NAM 2.5mM
H2O2+ NAM 5mM
NAM 5mM +H2O2

Figure-14: Effect of Nicotinamide post treatment on IGF-1 and SASP mRNA expression. RNA was extracted from NT, H_2O_2, and NAM-treated cells, and RT-qPCR was conducted. The mean ± S.E. relative mRNA expression of (A) IGF-1, (B) IL-6, (C) IL-8, and (D) TNF-alpha is represented in the data. The One-Way ANOVA and Tukey's post-hoc test was used to compare groups. If the P value was less than 0.05, differences between samples were considered significant. $P < 0.0001$ (####) differences between NT and H_2O_2 treated cells; no significant (ns) differences between H_2O_2 and H_2O_2+ NAM treated group; $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****) differences between H_2O_2 and NAM+ H_2O_2 groups.

Discussion

Cellular senescence is a condition that occurs as a range of stimuli and results in an irreversible cell cycle arrest. Senescent cells accumulate as people get older because the microtissue environment. When stress is created, the cells undergo a growth arrest phase. This has been linked to a number of age-related disorders (47). According to the literature, DNA repair processes become less effective as people become older. If DNA damage is not repaired, it can lead to cellular senescence, which makes it difficult for injured tissues to heal. Related to IGF-1, senescence fibroblasts produce lower levels of this hormone which then results in keratinocytes being at increased risk for malignant degeneration (22).

Previously our group has confirmed that pre-treatment with creatine monohydrate and nicotinamide shows a protective effect on stress-induced senescence by decreasing the percentage of beta galactosidase positive cells, increasing the production of IGF-1 and decreasing SASP mRNA expression. Based on these studies, the present work was designed to address two separate questions. First, do these pro-energetics block the increased cellular ROS in response to a pro-oxidative stressor. Second, we wanted to know if the post-treatment shows the similar effect as pre-treatment.

In our research, hydrogen peroxide (H_2O_2) is used to trigger senescence by inducing stress. According to the literature, H_2O_2 has been shown to enhance superoxide ions and free radicals, which raises ROS levels in cells. This is the most common cause of DNA damage. Experiments have been carried out to see if post-treatment with creatine and nicotinamide lowers H_2O_2-induced ROS production in HDF's. We found that the cells treated with 600 μM H_2O_2 showed a significant increase in ROS levels compared to no treatment. Whereas creatine +H_2O_2, nicotinamide +H_2O_2 and combination treatment decrease the ROS levels relative to the H_2O_2 treated group.

One of the biomarkers of cellular senescence is senescence-associated beta-galactosidase (SA-β-gal or SABG). It is a putative hydrolase enzyme that exclusively occurs in senescent cells that catalyzes the hydrolysis of β-galactosidase into monosaccharides (48). Our data was able to show that the cells treated with 600 μM H_2O_2 showed a significant rise in beta-galactosidase positive cells when compared to the no treatment group but there is no significant difference between H_2O_2 treated cells compared to H_2O_2+ creatine, H_2O_2+ nicotinamide and combination treated group.

Reduced skin IGF-1 expression in geriatric persons is due to age-related fibroblast senescence. Our research shows that the cells treated with 600 μM H_2O_2 showed a substantial drop in IGF-1 mRNA expression compared to no treatment, while the post-treatment with creatine, nicotinamide and combination groups had no effect in H_2O_2-induced senescence.

Senescent cells have an active metabolism and produce a lot of SASP factors. These factors include growth factors, inflammatory cytokines, proteases and immune modulators. We performed RT-qPCR to measure the mRNA levels of IL-6, IL-8 and TNF-α (important SASP factors). Based on our findings, post-treatment with creatine, nicotinamide and combination treatment groups does not show significant difference in SASP factors (IL-6, IL-8 and TNF-α) compared to H_2O_2 treated groups.

The two findings from the present studies fit together nicely, as it would make sense that if the mechanism for pro-energetic protection against oxidant-induced senescence involve decreasing cellular ROS, that treatment post oxidative stressor would have no effect based on the beta gal, IGF-1 and SASP mRNA expressions. Translating these findings into the clinic would suggest that these agents would be more of a preventative rather than a treatment for aged skin.

Future studies

For future studies, we can test the effect of creatine and nicotinamide in murine models, skin explants and human subjects. The first set of studies are currently ongoing, which are to test the ability of creatine and nicotinamide on replication-induced senescence in fibroblasts. Yet, given our findings that these agents serve as a preventative rather than a treatment, consideration could be given to investigate the effect of agents that actually remove senescent cells (senolytics) in human dermal fibroblasts.

Conclusion

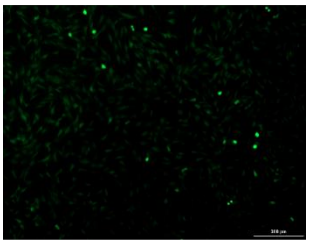
In conclusion, the results of the present study demonstrated that pretreatment with creatine and nicotinamide decrease the levels of ROS in oxidant stress-induced senescence dermal fibroblasts. Whereas post-treatment with creatine and nicotinamide does not show any effect on oxidant stress-induced senescence in dermal fibroblasts.

Supplementary Data

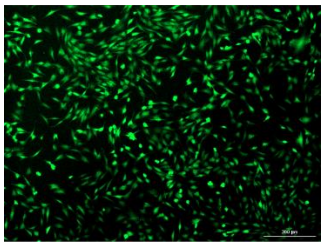
Creatine/Nicotinamide decreases H_2O_2-induced ROS accumulation in TERT cells.

In this study, we used the DCFH2-DA fluorescent method to measure intracellular ROS levels in H_2O_2-exposed N/TERT [Telomerase reverse transcriptase (TERT)–immortalized primary cells] cells. As shown in Figure-15, the H_2O_2-treated group drastically increased intracellular ROS when compared to the NT (No Treatment) group, however NAM 5 mM + H_2O_2, Cr 10 mM + H_2O_2, and combination therapy (Cr 5 mM + NAM 5 mM + H_2O_2) significantly decreased ROS levels.

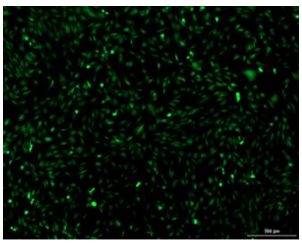
(A)



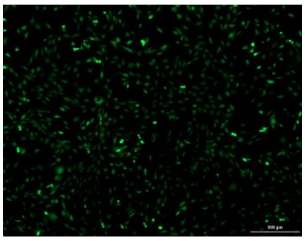
NT



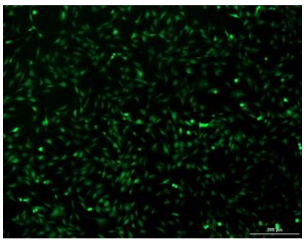
H_2O_2 600 µM



N 5mM +H_2O_2



C 10mM +H_2O_2



C 5mM + N 5mM +H_2O_2

(B)

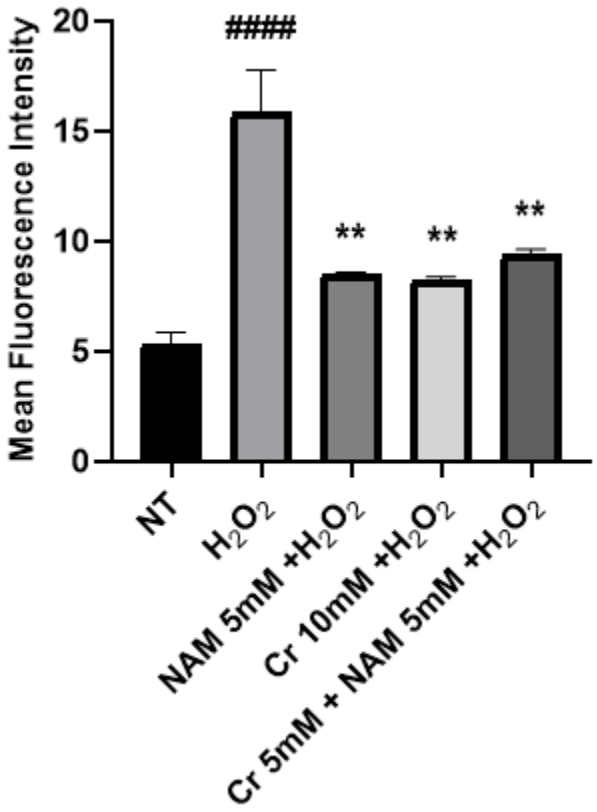


Figure-15: Creatine/Nicotinamide reduces H_2O_2-induced ROS production in N/TERT cells. N/TERT immortalized HDF's cells were incubated with or without Cr/NAM (5mM, 10mM) for 24 hrs. After the cells were washed and incubated with 5 µM DCFH2–DA for 30 mins, followed by 30mins 600 µM H_2O_2 treatment. Fluorescence intensity was measured by using image J software. The data is shown as the mean ± SE of three experiments. One-way ANOVA and Tukey's post-hoc test was used to compare groups. $P < 0.0001$ (####) compared with NT grouped cells, ($P < 0.01$**) compared with H_2O_2 treated cells.

Creatine/Nicotinamide increases SOD activity in H2O2-induced TERT cells.

In this study, SOD Assay kit-WST (Dojindo Molecular Technologies) was used to evaluate SOD activity in H_2O_2-exposed N/TERT [Telomerase reverse transcriptase (TERT)–immortalized primary cells] cells. Cells were incubated with or without Cr/NAM (2.5mM, 5mM, 10mM) for 24 hrs. After the cells were washed and incubated with 600 µM H_2O_2 treatment for 30 mins. Collect the cells after washing them for 2-3 times and centrifugation was performed to collect the supernatant. Then perform the experiment according to the manufacturer's instructions from the assay kits. As shown in Figure-16, the H_2O_2-treated group drastically decreased SOD activity when compared to the NT (No Treatment) group, however Cr 5 mM + H_2O_2, Cr 10 mM + H_2O_2, NAM 2.5 mM + H_2O_2, NAM 5 mM + H_2O_2 and combination therapy (Cr 5 mM + NAM 5 mM + H_2O_2 significantly increases the SOD activity in fibroblasts in comparison with those in the H2O2 treated cells.

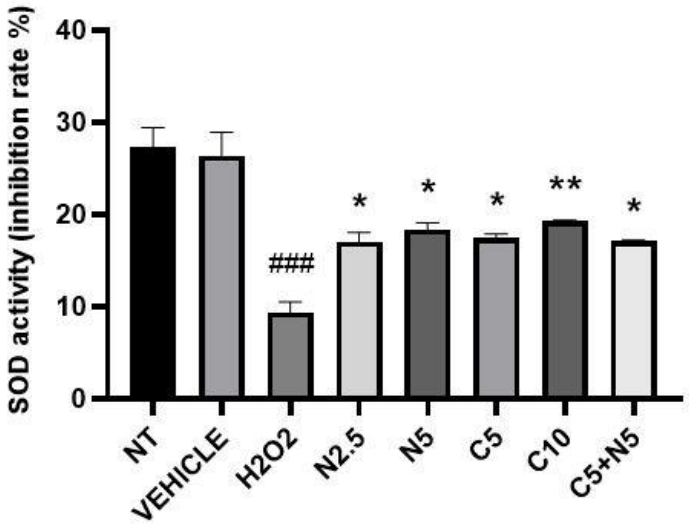


Figure-16: Effect of creatine/nicotinamide on changes of SOD. Cells were incubated with or without Cr/NAM (2.5mM, 5mM, 10mM) for 24 hrs. After the cells were washed and incubated with 600 µM H_2O_2 treatment for 30 mins. The data is shown as the mean ± SE of two experiments. One-way ANOVA and Tukey's post-hoc test was used to compare groups. $P < 0.001$ (###) compared with NT grouped cells, ($P < 0.05$*, $P < 0.01$**) compared with H_2O_2 treated cells.

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